THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 38, pp. 29387–29397, September 17, 2010

Phosphorylation of a Novel Cytoskeletal Protein (RsmP) Regulates Rod-shaped Morphology in *Corynebacterium glutamicum**

Received for publication, June 14, 2010, and in revised form, July 7, 2010 Published, JBC Papers in Press, July 9, 2010, DOI 10.1074/jbc.M110.154427

Maria Fiuza[‡1], Michal Letek[‡], Jade Leiba[§], Almudena F. Villadangos[‡], José Vaquera[‡], Isabelle Zanella-Cléon[§], Luís M. Mateos[‡], Virginie Molle[§2], and José A. Gil[‡3]

From the [‡]Departamento de Biología Molecular, Área de Microbiología, Facultad de Biología, Universidad de León, León 24071, Spain and the [§]Institut de Biologie et Chimie des Protéines (UMR 5086), CNRS, Université Lyon1, IFR128 BioSciences, Lyon-Gerland, 7 Passage du Vercors, 69367 Lyon Cedex 07, France

Corynebacteria grow by wall extension at the cell poles, with DivIVA being an essential protein orchestrating cell elongation and morphogenesis. DivIVA is considered a scaffolding protein able to recruit other proteins and enzymes involved in polar peptidoglycan biosynthesis. Partial depletion of DivIVA induced overexpression of *cg3264*, a previously uncharacterized gene that encodes a novel coiled coil-rich protein specific for corynebacteria and a few other actinomycetes. By partial depletion and overexpression of Cg3264, we demonstrated that this protein is an essential cytoskeletal element needed for maintenance of the rod-shaped morphology of *Corynebacterium glutamicum*, and it was therefore renamed RsmP (rod-shaped morphology protein). RsmP forms long polymers *in vitro* in the absence of any cofactors, thus resembling eukaryotic intermediate filaments. We also investigated whether RsmP could be regulated post-translationally by phosphorylation, like eukaryotic intermediate filaments. RsmP was phosphorylated *in vitro* by the PknA protein kinase and to a lesser extent by PknL. A mass spectrometric analysis indicated that phosphorylation exclusively occurred on a serine (Ser-6) and two threonine (Thr-168 and Thr-211) residues. We confirmed that mutagenesis to alanine (phosphoablative protein) totally abolished PknA-dependent phosphorylation of RsmP. Interestingly, when the three residues were converted to aspartic acid, the phosphomimetic protein accumulated at the cell poles instead of making filaments along the cell, as observed for the native or phosphoablative RsmP proteins, indicating that phosphorylation of RsmP is necessary for directing cell growth at the cell poles.

In virtually all bacteria, the peptidoglycan (PG)[4] cell wall maintains the shape and integrity of the cell. However, it is less clear how the shape of the cell wall "sacculus," and thereby the cell itself, is genetically determined to yield a specific morphology. Recently, cytoskeletal proteins in the cytoplasm have been shown to be critical in determining bacterial cell shape and in controlling the two distinct modes of cell wall assembly, cell division and cell elongation, in time and space (for reviews see Refs. 1–3). Septa are organized and positioned at midcell by the tubulin homologue FtsZ, which forms a cytokinetic ring at the division site and recruits the various proteins (FtsA, FtsW, PBPs, etc.) involved in positioning the division septum at this site (4). Each division event gives rise to two daughter cells that undergo cell elongation. In most of the well established rod-shaped models such as *Escherichia coli* (5), *Bacillus subtilis* (6), and *Caulobacter crescentus* (7), cell elongation occurs by intercalation of new PG into the lateral wall along most of its length, and the poles remain largely inert (8). This elongation requires the actin homologue MreB, which assembles into a helical cytoskeleton along the cell (9). Daniel and Errington (10) used fluorescently labeled vancomycin (Van-FL) staining to visualize active sites of PG assembly and showed that elongation of the lateral wall occurred by insertion of new cell wall material in a helical pattern along the length of the cell and that this pattern was dependent on the MreB isoform Mbl. Together, these observations led to a model in which the helical MreB cytoskeleton plays a role in organizing or localizing enzymes involved in cell wall assembly during elongation of the lateral wall, presumably by being linked to the cell wall synthetic machinery via MreC, MreD, and RodA (1, 11–13).

Although most rod-shaped or filamentous bacteria possess *mreB* genes, a second MreB-independent mode of cell elongation and acquisition of rod shape is present in actinobacteria, like *Corynebacterium glutamicum*, *Streptomyces coelicolor*, and *Mycobacterium tuberculosis*. In agreement with a previous report in *Corynebacterium diphtheriae* (14), staining with Van-FL revealed in *C. glutamicum* assembly of PG primarily at the cell poles instead of along the lateral wall (10). Because corynebacterial genomes lack *mreB* homologues (15–18), this polar cell wall elongation must be MreB-independent. Thus, in the simplest model for polarization of PG assembly in *C. glutamicum*, components of the cell division machinery would be sufficient to recruit the enzymes for wall elongation to the new cell pole. Interestingly, recent reports in *C. glutamicum*, *S. coelicolor*, and *M. tuberculosis* demonstrated that DivIVA is involved in apical growth and cell shape determination in these

* This work was supported in part by National Research Agency Grants ANR-06-MIME-027-01 and ANR-09-MIEN-004 (to V. M.), Junta de Castilla y León Grants LE040A07 and LE28A10-2 (to J. A. G. and L. M. M.), and Ministerio de Ciencia y Tecnología (Spain) Grant BIO2008-00519 (to J. A. G.).

[1] Recipient of a fellowship from the Ministerio de Educación y Ciencia.

[2] To whom correspondence may be addressed. Tel.: 33-4-72-72-26-79; Fax: 33-4-72-72-26-41; E-mail: vmolle@ibcp.fr.

[3] To whom correspondence may be addressed. Tel.: 34-987-29-15-03; Fax: 34-987-29-14-09; E-mail: jagils@unileon.es.

[4] The abbreviations used are: PG, peptidoglycan; DTBP, dimethyl 3,3′-dithiobispropionimidate; IF, intermediate filament; Van-FL, fluorescently labeled vancomycin; STPK, serine/threonine protein kinase.

TABLE 1
Bacterial strains and plasmids used in this study

Strains or plasmids	Genotype or description	Source or Ref.
E. coli TOP10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL endA1 nupG*; used for general cloning	Invitrogen
E. coli S17-1	Mobilizing donor strain, *pro recA*, with an RP4 derivative integrated into the chromosome	71
E. coli BL21(DE3)Star	F2 *ompT hsdSB*(rB2 mB2) *gal dcm* (DE3); used to express recombinant proteins in *E. coli*	Stratagene
C. glutamicum ATCC 13869	Wild-type control strain	ATCC
C. glutamicum R31	*C. glutamicum* ATCC 13869; derivative used as recipient in conjugations	72
C. glutamicum LACID	*C. glutamicum* R31 derivative carrying a chromosomal copy of *divIVA* under the control of P*lac* and a plasmid carrying *lacI*^{q}	23
C. glutamicum LAC3264	*C. glutamicum* R31 derivative carrying a chromosomal copy of *cg3264* under the control of P*lac* obtained by integration of plasmid pOJ3264	This work
pOJ260	Mobilizable plasmid containing an *E. coli* origin of replication and the apramycin resistance gene (*am*)	42
pOJ3264int	pOJ260 derivate carrying an internal 419-bp fragment of the *cg3264/rsmP* gene from *C. glutamicum* ATCC 13869	This work
pOJ3264	pOJ260 derivate carrying the 5′ end (667-bp) of the *cg3264/rsmP* gene from *C. glutamicum* under the control of the P*lac* promoter	This work
pEAG6	Mobilizable plasmid able to replicate in *E. coli* and *C. glutamicum* containing the P*div* promoter, *egfp2,* and the kanamycin resistance gene (*kan*)	23
pE_RsmP-GFP	pEAG6 derivative carrying the wild-type *rsmP* gene and expressing the wild-type RsmP protein fused to GFP	This work
pE_RsmPA-GFP	pEAG6 derivate carrying an *in vitro* mutated *rsmP* gene and expressing a RsmP protein with S6A/T168A/T211A and fused to GFP	This work
pE_RsmPD-GFP	pEAG6 derivate carrying an *in vitro* mutated *rsmP* gene and expressing a RsmP protein with S6D/T168D/T211D and fused to GFP	This work
pETPhos	pETEV derivative used to express His-tagged proteins	73
pPhosRsmP	pETPhos derivative used to express His-tagged wild-type RsmP protein	This work
pPhosRsmP_S6A	pPhosRsmP derivative used to express His-tagged RsmP protein S6A	This work
pPhosRsmP_T168A	pPhosRsmP derivative used to express His-tagged RsmP protein T168A	This work
pPhosRsmP_T211A	pPhosRsmP derivative used to express His-tagged RsmP protein T211A	This work
pPhosRsmP_S6D	pPhosRsmP derivative used to express His-tagged RsmP protein S6D	This work
pPhosRsmP_2A	pPhosRsmP_S6A derivative used to express His-tagged RsmP protein S6A/T168A	This work
pPhosRsmP_2D	pPhosRsmP_S6D derivative used to express His-tagged RsmP protein S6D/T168D	This work
pPhosRsmP_3A	pPhosRsmP_2A derivative used to express His-tagged RsmP protein S6A/T168A/T211A	This work
pPhosRsmP_3D	pPhosRsmP_2D derivative used to express His-tagged RsmP protein S6D/T168D/T211D	This work
PGEXA	pGEX4T-3 derivative used to express GST fusion of PknA cytoplasmic domain	33
pGEXB	pGEX4T-3 derivative used to express GST fusion of PknB cytoplasmic domain	33
pGEXL	pGEX4T-3 derivative used to express GST fusion of PknL cytoplasmic domain	33

organisms (19–23). The DivIVA proteins are predicted to have a high proportion of coiled-coil structure and able to form oligomers with a filamentous structure required for DivIVA function (24–26).

Actin, tubulin, and intermediate filaments (IFs) constitute the eukaryotic cytoskeleton, responsible, among others factors, for cell shape in higher organisms. Actin-like (MreB, ParM, and MamK) and tubulin-like (FtsZ) proteins have been identified in bacteria and are considered responsible for the multitude of cell shapes encountered in the prokaryotes (27). However, less information is available concerning IF-like elements in bacteria. The first IF protein described in prokaryotes was crescentin (CreS) (28), responsible for the specific cell shape of *C. crescentus* as mutants lacking CreS showed straight-rod morphology. Bagchi *et al.* (29) demonstrated the existence of another IF protein (FilP or SCO5396) forming cytoskeletal structures in *S. coelicolor,* which were involved in the mechanical strength of the hyphae and required for normal growth and morphogenesis. In addition, Waidner *et al.* (30) showed that the helical shape of the human pathogen *Helicobacter pylori* was dependent on coiled-coil-rich proteins (Ccrp59 and Ccrp1143), which form filamentous structures *in vitro* and *in vivo*. Interestingly, CreS, FilP, and eukaryotic IF proteins share a central "rod domain" of four alternating coiled-coil segments and linkers, flanked by more globular head (N-terminal) and tail (C-terminal) domains, with the rod domain being essential for filament formation (31). In contrast, Ccrps are almost entirely composed of coiled coils and lacks the characteristic head and tail domains (30). The most important feature of coiled-coil domains appears to be their ability to act as a "cellular Velcro" to hold together molecules, subcellular structures, and even tissues in higher organisms.

In this study, we identified and characterized a novel cytoskeletal protein that constitutes an essential component of the corynebacterial cytoskeleton and that is also present in all corynebacterial genomes already sequenced, as well as in a few number of actinomycetes. The protein, originally named as Cg3264 and now renamed RsmP (rod-shaped morphology protein) for its role in rod shape determination, is essential for *C. glutamicum* viability, can form filaments both *in vivo* and *in vitro*, and is regulated via serine/threonine kinase phosphorylation. To our knowledge, this work represents the first evidence of a corynebacterial cytoskeletal protein regulated by phosphorylation, thus demonstrating that *C. glutamicum* possesses an original and specific system for establishing and maintaining rod-shaped morphology.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Conjugal Plasmid Transfer from E. coli to C. glutamicum—Bacterial strains and plasmids are described in Table 1. Strains used for cloning and expression of recombinant proteins were *E. coli* TOP10 (Invitrogen) and *E. coli* BL21(DE3)Star (Stratagene), respectively. *E. coli* was grown and maintained at 37 °C in LB medium

TABLE 2
Primers used in this study
Restriction sites are underlined. Mutated codons are in boldface.

Primer	5′ to 3′ Sequence[a]
cg3264int1	CGGAATTCAGATAAGTCCGCAAC (EcoRI)
cg3264int2	CTATAAGCTTCGGACTGCGCAATC (HindIII)
3264lac1	CGGAATTCAATGGCTAATCCGC (EcoRI)
cg3264-1	GGAATTCCATATGGCTAATCCGCTCAGCAAG (NdeI)
cg3264-2ns	GGAAT TCCATATGTTTCTTCTCGGACTC (NdeI)
cg3264-2	CGGGATCCTTATTTCTTCTCGGACTC (BamHI)
N-RsmP6A	ATG GCT AAT CCG CTC **GCC** AAG GGC TGG AAG TAT CTC
C-RsmP6A	GAG ATA CTT CCA GCC CTT **GCC** GAG CGG ATT AGC CAT
N-RsmP6D	ATG GCT AAT CCG CTC **GAC** AAG GGC TGG AAG TAT CTC
C-RsmP6D	GAG ATA CTT CCA GCC CTT **GTC** GAG CGG ATT AGC CAT
N-RsmP168A	AAG ATG CAG GAA AGT GTC **GCT** AAG TCT ATG GAT TCT TTG
C-RsmP168A	CAA AGA ATC CAT AGA CTT **AGC** GAC ACT TTC CTG CAT CTT
N-RsmP168D	AAG ATG CAG GAA AGT GTC **GAT** AAG TCT ATG GAT TCT TTG
C-RsmP168D	CAA AGA ATC CAT AGA CTT **ATC** GAC ACT TTC CTG CAT CTT
N-RsmP211A	CAG GAA CTT ACC CAG AAC **GCT** GTT AGT GAT CGC ATG GCT
C-RsmP211A	AGC CAT GCG ATC ACT AAC **AGC** GTT CTG GGT AAG TTC CTG
N-RsmP211D	CAG GAA CTT ACC CAG AAC **GAT** GTT AGT GAT CGC ATG GCT
C-RsmP211D	AGC CAT GCG ATC ACT AAC **ATC** GTT CTG GGT AAG TTC CTG

supplemented with 100 μg/ml ampicillin, 50 μg/ml apramycin, or 50 μg/ml kanamycin when required. *C. glutamicum* was grown at 30 °C in trypticase soy broth (Oxoid) or trypticase soy broth containing 2% agar medium supplemented with 12.5 μg/ml apramycin and/or 12.5 μg/ml kanamycin when needed. Plasmids to be transferred by conjugation from *E. coli* to corynebacteria were introduced by transformation into the donor strain *E. coli* S17-1. Mobilization of plasmids from *E. coli* S17-1 to *C. glutamicum* R31 was accomplished as described previously (32).

Two-dimensional Gel Electrophoresis—Wild-type *C. glutamicum* R31 and LACID strains (Table 1) were grown to early stationary phase. Cells were harvested, washed twice with 10 mM Tris-HCl, pH 8, and resuspended in lysis buffer (10 mM Tris-HCl, pH 8, 5 mM EDTA, and 100 mM DTT), and antiprotease mixture (Roche Applied Science). *C. glutamicum* was disrupted using Fast PROTEIN Blue Lysing Matrix (Qbiogene, Carlsbad, CA, USA) and the BIO101 Thermo Savant FastPrep FP120 (Qbiogene Inc.). The lysate was cleared by centrifugation at 14,000 rpm for 30 min at 4 °C. Approximately 150 μg of total soluble proteins were loaded onto a 7-cm Immobiline^{TM} strip (Bio-Rad, pH 4–7) and electrophoresed in a Protean IEF cell in the first dimension and on a 10% SDS-PAGE in the second dimension. Proteins were identified by mass spectrometry using an Ultraflex III MALDI-TOF (Bruker Daltonics) and the software Flex-Analysis and Biotools (Bruker Daltonics).

Construction of Plasmids for C. glutamicum Manipulations—An internal fragment of *cg3264* was amplified from the *C. glutamicum* ATCC 13869 chromosome using primers cg3264int1/cg3264int2 (Table 2). The amplified 419-bp fragment was EcoRI/HindIII-digested and subcloned in the suicide plasmid pOJ260, yielding pOJ3264int (Table 1). To reduce expression of *cg3264* in *C. glutamicum*, a plasmid was designed to place the copy of *cg3264* under the control of the P*lac* promoter. The first 667 bp of *cg3264* were amplified by PCR using 3264lac1/3264int2 primers (Table 2), digested with EcoRI and HindIII, and subcloned in the pOJ260 plasmid, yielding pOJ3264 (Table 1). Genetic constructs of *C. glutamicum* transconjugant strains were confirmed by PCR and Southern blot hybridization, using probes obtained by PCR amplification and labeled with digoxigenin according to the manufacturer's instructions (Roche Applied Science). To study localization of the Cg3264/RsmP protein in *C. glutamicum, cg3264* mutated gene copies were PCR-amplified without stop codons using the cg3264-1/cg3264-2ns primer pair. The PCR product was NdeI-digested and cloned into the bifunctional mobilizable vector pEAG6, yielding the vectors pE_RsmP-GFP, pE_RsmPA-GFP, and pE_RsmPD-GFP (Table 1). The pEAG6 vector was used to clone any gene under control of the P*div* promotor and fused to the *egfp2* gene (encoding an enhanced green fluorescent protein) (23).

Cloning, Expression, and Purification of Cg3264 Proteins—First, *cg3264* was cloned to generate a recombinant protein expressed in *E. coli*. Then *cg3264* was amplified by PCR using *C. glutamicum* ATCC 13869 genomic DNA as a template and the primer pair cg3264-1/cg3264-2 (Table 2), containing NdeI and BamHI restriction sites, respectively. The 876-bp amplified product was digested with NdeI and BamHI and ligated with the pETPhos vector generating pPhosRsmP (Table 1). *E. coli* BL21(DE3)Star cells transformed with this construct were used for expression and purification of His_6-tagged RsmP under native conditions with nickel-nitrilotriacetic acid resin (Qiagen) as described previously (33, 34). To purify the protein under denaturing conditions, 8 M urea was used as described in the Qiagen user manual.

Production of Antibodies against RsmP and Protein Quantification—The native His-tagged RsmP was used to immunize male rabbits (Speedy 28-day Rabbit Programme, Eurogentec) for the production of polyclonal anti-RsmP antibodies. The resulting polyclonal antibodies specifically recognized a protein of 43 kDa, the expected size of RsmP. To quantify RsmP, total proteins from *C. glutamicum* were quantified by Bradford. Proteins (1 μg) were separated by SDS-PAGE and stained with Coomassie Blue or electroblotted onto polyvinylidene difluoride membranes (Millipore); the latter was immunostained with a 1:10,000 dilution of rabbit polyclonal antibodies raised against His tags (Santa Cruz Biotechnology) and with polyclonal antibodies raised against purified His-tagged RsmP (see above). Anti-rabbit immunoglobulin G-alkaline phosphatase

(Santa Cruz Biotechnology) was used as the secondary antibody at a 1:10,000 dilution.

In Vitro Filament Formation and Electron Microscopy—*In vitro* polymerization experiments were performed using the cross-linking agent dimethyl 3,3′-dithiobispropionimidate (DTBP) (Pierce). The proteins purified under native conditions in 50 mM NaH_2PO_4 and 300 mM NaCl buffer, were incubated with 10 times molar excess of DTBP for 15 or 30 min at room temperature. The polymerization reaction is reversible by the addition of 1 mM dithiothreitol at 37 °C for 30 min. To stop the reaction, 1 M Tris-HCl, pH 8.0, was added. Proteins were separated on a 12% SDS gel, electroblotted onto polyvinylidene difluoride membranes (PVDF, Millipore), and detected using anti-His antibodies at 1:10,000. Alkaline phosphatase antibody-conjugated anti-rabbit was used as a secondary antibody at a 1:10,000 dilution. To induce filament formation, protein samples purified under denaturing conditions were dialyzed against a buffer containing 10 mM Tris-HCl with 150 mM NaCl at pH 7.0, overnight at 4 °C. The dialyzed samples were applied to carbon-coated grids, stained with 1% uranyl acetate, and observed by transmission electron microscopy.

In Vitro Kinase Assays—*In vitro* phosphorylation was performed with 2 μg of RsmP in 20 μl of buffer P (25 mM Tris-HCl, pH 7, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA) with 200 μCi/ml [γ-^{33}P]ATP corresponding to 65 nM (PerkinElmer Life Sciences, 3000 Ci/mmol) and 0.5 μg of kinase. Plasmids pGEXA, pGEXB, and pGEXL (Table 1) were used for expression and purification in *E. coli* of the recombinant STPKs from *C. glutamicum* as described previously (33). After a 15-min incubation, the reaction was stopped by adding sample buffer and heating the mixture at 100 °C for 5 min. The reaction mixtures were analyzed by SDS-PAGE. After electrophoresis, gels were soaked in 20% trichloroacetic acid for 10 min at 90 °C, stained with Coomassie Blue, and dried. Radioactive proteins were visualized by autoradiography using direct exposure films.

Mass Spectrometry Analysis—Purified wild-type and mutant RsmP proteins were subjected to *in vitro* phosphorylation by GST-tagged PknA as described above, except that [γ-^{33}P]ATP was replaced with 5 mM ATP. Subsequent analyses using nanoLC/nanospray/tandem mass spectrometry (LC-ESI/MS/MS) were performed as described previously (33).

Site-directed Mutagenesis—The three residues from *C. glutamicum* RsmP identified by mass spectrometry after *in vitro* phosphorylation with GST-tagged PknA were independently replaced by alanine residues by site-directed mutagenesis using inverse PCR amplification. A first PCR was carried out using pPhosRsmP (Table 1) as a template with the primer pair N-RsmP6A/C-RsmP6A (Table 2) to generate pPhosRsmP_S6A (S6A). The second and third mutants were obtained using pPhosRsmP as template with the primer pairs N-RsmP168A/C-Rsm168A and N-RsmP211A/C-Rsm211A (Table 2) to generate pPhosRsmP_T168A (T168A) and pPhosRsmP_T211A (T211A), respectively (Table 1). pPhosRsmP was also used as template with the primer pair N-RsmP6D/C-RsmP6D (Table 2) to replace the serine 6 by aspartic acid yielding pPhosRsmP_S6D (S6D) (Table 1). A new PCR was carried out using pPhosRsmP_S6A and pPhosRsmP_S6D plasmids (Table 1) as template with the primer pairs N-RsmP168A/C-Rsm168A and N-RsmP168D/C-RsmP168D (Table 2) to generate pPhosRsmP_2A (S6A/T168A) and pPhosRsmP_2D (S6D/T168D). These mutants were used as a template in another PCR using the primer pairs N-RsmP211A/C-Rsm211A and N-RsmP211D/C-RsmP211D (Table 2), yielding pPhosRsmP_3A (S6A/T168A/T211A) and pPhosRsmP_3D (S6D/T168D/T211D), respectively. All the resulting constructs were verified by DNA sequencing. The different His-tagged mutant proteins were overexpressed and purified as described above.

Microscopy—Living *C. glutamicum* or stained cells with fluorescent dyes were observed in a Nikon E400 fluorescence microscope. Pictures were taken with a DN100 Nikon digital camera. Vancomycin BODIPY FL (Van-FL, Molecular Probes) staining was performed by adding an equal portion of unlabeled vancomycin and Van-FL to growing cultures at a final concentration of 1 μg/ml (10). The culture was then incubated for 5 min to allow adsorption of the antibiotic, after which the cells were viewed directly by fluorescence microscopy. Staining with 4′,6-diamino-2-phenylindole (DAPI) was performed as described previously (35).

RESULTS

Coccoid C. glutamicum DivIVA-depleted Cells Showed Increased Expression of a Novel Coiled-coil Protein—Previously, we constructed a *C. glutamicum* LACID strain, expressing low levels of DivIVA (23). This strain presented a coccoid phenotype that was morphologically different from the rod-shaped cells of the parental strain *C. glutamicum* R31. The total cytoplasmic proteins synthesized by *C. glutamicum* R31 (parent strain) and *C. glutamicum* LACID (DivIVA partially depleted *C. glutamicum,* Table 1) were characterized by two-dimensional gel electrophoresis; representative gels depicting a consistent pattern of the protein profiles are shown in Fig. 1*A*. Fifteen proteins of various molecular sizes appeared to be over- or underexpressed in *C. glutamicum* LACID compared with the R31 parental strain (Fig. 1*B*).

Among the 13 underexpressed proteins, 10 could be identified by peptide mass mapping technique as alkyl hydroperoxidase (Cg2674), isocitrate lyase (Cg2560), CoA-transferase (Cg2623), *N*-acetylglutamate synthase (Cg2382), fructose-biphosphate aldolase (Cg3068), phosphoglyceromutase (Cg0482), thioredoxin domain-containing protein (Cg0792), phospho-3-sulfolactate synthase (Cg2797), MraZ (Cg2378), and a ferritin-like protein (Cg2782). Apart from MraZ, which is encoded by the first gene of the bacterial *dcw* (division and cell wall biosynthesis) cluster with its promoter (P*mra*) responsible for the transcription of nine genes of the cluster (36), the remaining identified proteins were involved in central metabolic pathways. These results indicate that partial depletion of DivIVA in *C. glutamicum* represses expression of *mraZ* and probably the expression of downstream genes involved in cell division and PG biosynthesis, which were not detected/identified in our two-dimensional gel analysis.

In contrast, three proteins appeared to be overexpressed in *C. glutamicum* LACID, but only one (Cg3264) was positively identified as a protein similar to the phage shock protein A (PspA, Cg2151). Antibodies raised against Cg3264 confirmed that this

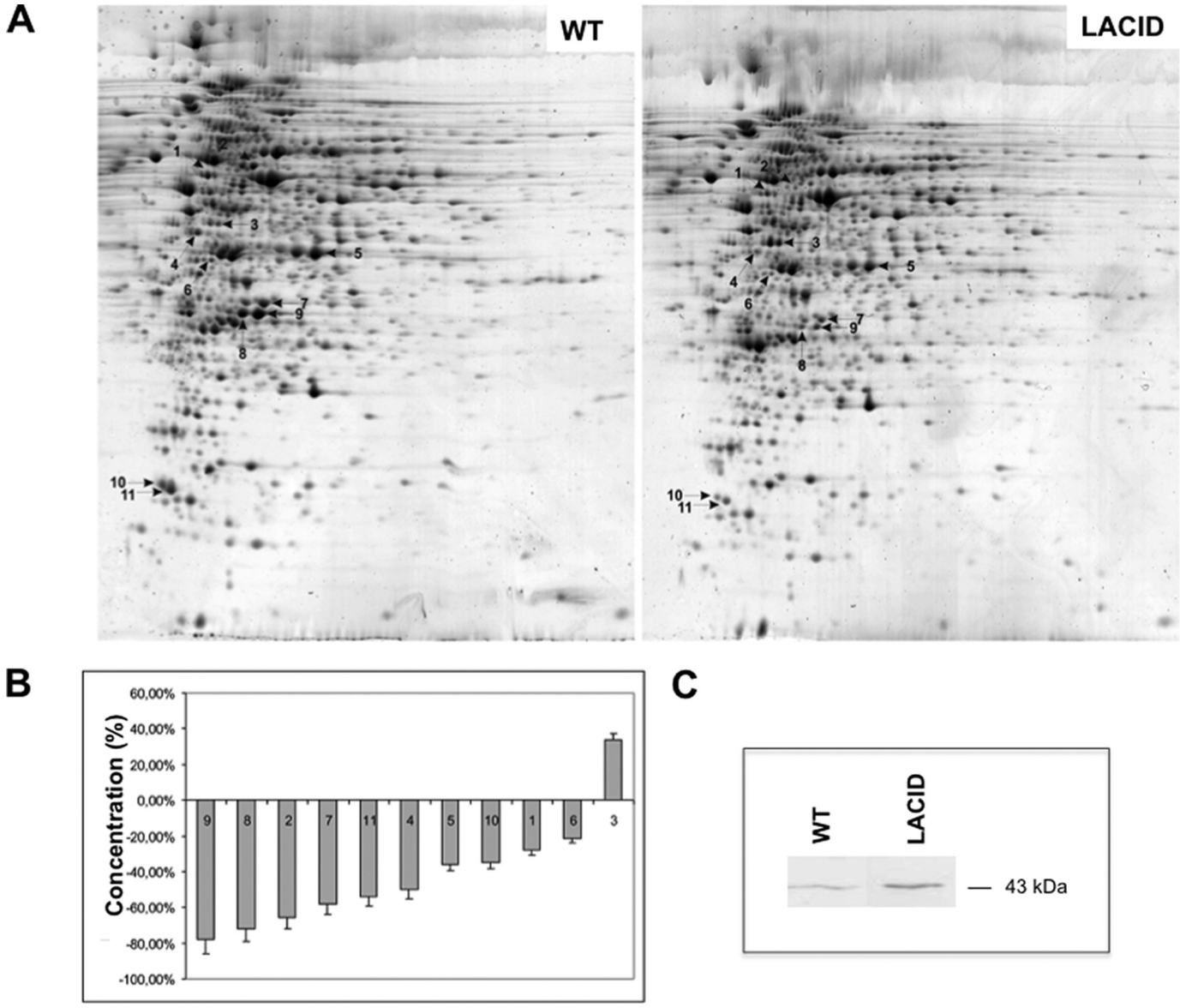

FIGURE 1. *A,* two-dimensional gel electrophoresis of proteins obtained from *C. glutamicum* R31 (wild type) and the partially depleted DivIVA strain (LACID). *Arrows* indicate proteins unequally expressed in WT and LACID strains. *B,* expression levels of unequally expressed protein between LACID and WT strains. *Bar 1,* alkyl hydroperoxidase (Cg2674); *bar 2,* isocitrate lyase (Cg2560); *bar 3,* RsmP (Cg3264); *bar 4,* CoA-transferase (Cg2623); *bar 5, N*-acetylglutamate synthase (Cg2382); *bar 6,* fructose-biphosphate aldolase (Cg3068); *bar 7,* phosphoglyceromutase (Cg0482); *bar 8,* thioredoxin domain-containing protein (Cg0792); *bar 9,* phospho-3-sulfolactate synthase (Cg2797); *bar 10,* MraZ (Cg2378); and *bar 11,* ferritin-like protein (Cg2782). *C,* RsmP expression levels. The various RsmP levels of the *C. glutamicum* R31 (wild type) strain and partially depleted DivIVA strain (LACID) were analyzed by immunoblotting using rabbit anti-RsmP antibodies. Cells were grown, harvested, and disrupted. Equal amounts (1 μg) of crude lysates were then loaded onto an acrylamide gel, subjected to electrophoresis, and transferred onto a membrane for immunoblot analysis.

protein was overexpressed in the LACID mutant (Fig. 1*C*). Both PspA and Cg3264 belong to the Pfam/InterPro family of PspA/IM30 (PF04012/IPR007157) proteins that putatively suppress σ54-dependent transcription (37) and could play a role in maintaining cytoplasmic membrane integrity and/or the proton-motive force (38). PspA and the PspA-like protein Cg3264 correspond to 2 of the 37 coiled-coil proteins present in the genome of *C. glutamicum*. Exhaustive analysis of the PspA/IM30 protein family in actinomycetes revealed authentic PspA proteins in all the sequenced actinomycete genomes, although Cg3264 homologues were found exclusively in corynebacterial genomes (Fig. 2) and not in those of *Mycobacterium* species. Only a few *Streptomyces* species (*Streptomyces* sp. Mg1, *Streptomyces griseus*, and *Streptomyces pristinaspiralis*) contained a Cg3264 homologue.

Interestingly, Cg3264 has a secondary structure rich in α-helical regions according to the program PSIPRED (39), which are predicted to contain a central rod domain of two coiled-coil segments (containing several heptad repeat regions) (Program PAIRCOIL (40)) as well as a linker lacking a coiled-coil structure (Fig. 3). Moreover, Cg3264 contains two stutters (41), where coiled-coil 2 is clearly interrupted for a few amino acids at the beginning and end of the coiled coil, and the rod domain is flanked by head and tail domains lacking the coiled-coil region and α-helical structures. Therefore, based on the facts that the Cg3264 protein is characteristic of corynebacte-

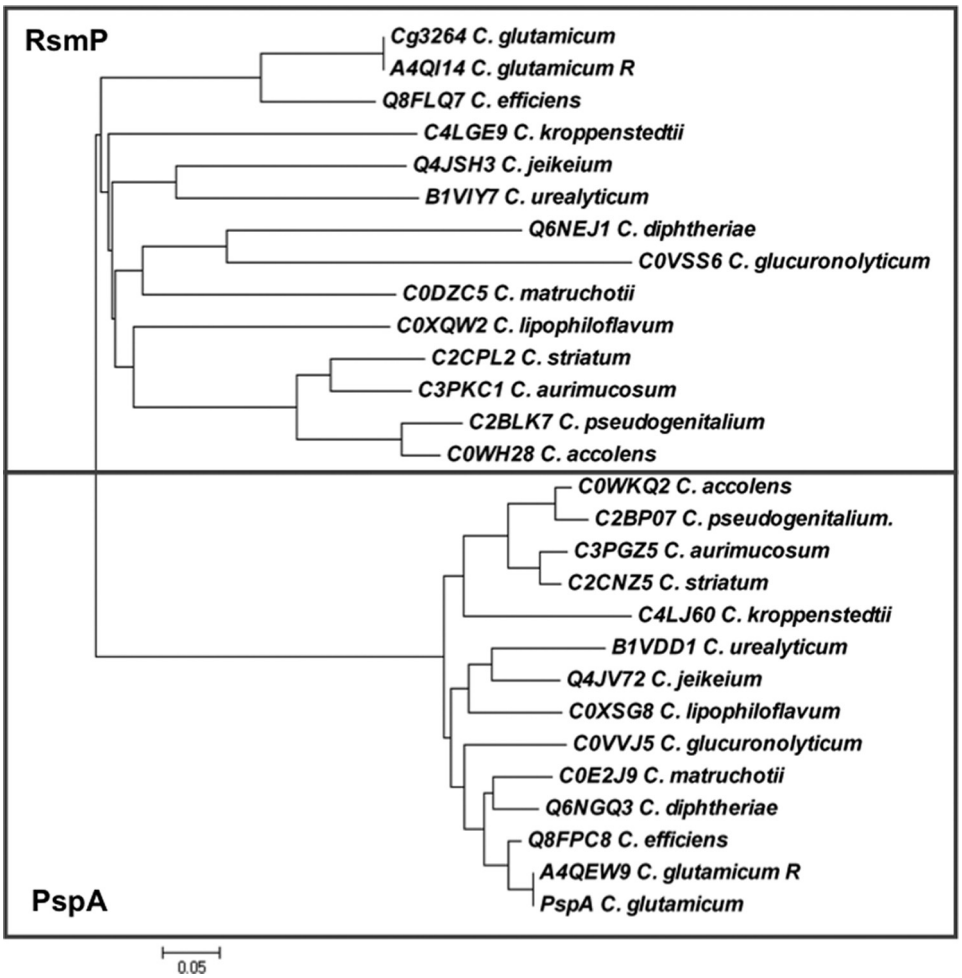

FIGURE 2. **Evolutionary relationships of PspA- and RsmP-like proteins in 14 complete corynebacterial genomes.** The evolutionary history was inferred using the Neighbor-Joining method (68). The optimal tree with the sum of branch lengths = 4.00209249 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (69) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were 218 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (70).

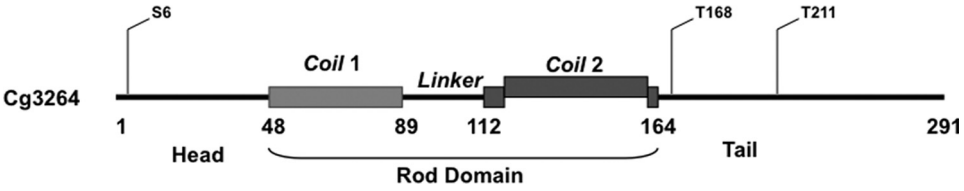

FIGURE 3. **Schematic representation of the head, rod domain, and tail regions of the wild-type RsmP protein from *C. glutamicum*.** The rod domain contains two coiled coils (*Coil 1* and *Coil 2*) separated by a linker; two discontinuities (stutters) are present in Coil 2. Phosphorylation sites of RsmP (Ser-6, Thr-168, and Thr-211) are indicated.

rial genomes and was overexpressed in the DivIVA-depleted *C. glutamicum* LACID strain, and that DivIVA is also a coiled-coil protein involved in polar growth (23), we hypothesized that the Cg3264 protein could also be involved in cell division or cell elongation in corynebacteria.

Cg3264 Is Essential and Involved in C. glutamicum Polar Growth—To investigate whether *cg3264* was necessary for the growth and viability of *C. glutamicum*, we attempted gene disruption experiments using an internal fragment of *cg3264* cloned into the conjugative suicide plasmid pOJ260 (42) yielding the pOJ-3264int vector (Table 1). This plasmid was introduced into *E. coli* S17-1 and the transformant mated with *C. glutamicum* R31. Apramycin-resistant transconjugants could not be obtained with pOJ-3264int after many attempts, suggesting that *cg3264* is essential for the viability of *C. glutamicum.* Because the genes located immediately downstream and upstream of *cg3264* are transcribed in the opposite direction to *cg3264*, a possible polar effect on adjacent gene expression by insertion of pOJ-3264int was discounted. We therefore constructed a conditional gene expression strain using the *Plac* promoter, which allows regulation of the expression of essential genes in *C. glutamicum* (43). *C. glutamicum* was transformed with the suicide plasmid pOJ3264 (P*lac-cg3264*) (Table 1), which, when introduced into *C. glutamicum,* could integrate by homologous recombination into the chromosomal *cg3264* locus, thus disrupting the *cg3264* gene under its natural promoter and creating a full-length copy under control of the P*lac* promoter. Southern blot analysis of DNA from the transconjugant strain *C. glutamicum* LAC3264 (Table 1) showed the expected pattern for Campbell-type integration of pOJ3264 at the chromosomal *cg3264* locus (data not shown).

The phenotype of this conditional mutant was investigated to define the function of the Cg3264 protein. Interestingly, the coccoid morphology of this mutant seems to be similar to the one obtained when the level of the essential protein DivIVA was depleted (Fig. 4*A*) (23), and the typical polar and septal Van-FL staining shown by the wild-type *C. glutamicum* R31 (23) was lost in the *C. glutamicum* LAC3264 strain (data not shown). On the other hand, antibodies raised against Cg3264 confirmed that this protein was underexpressed in *C. glutamicum* LAC3264 (Fig. 4*B*). Therefore, Cg3264, previously of unknown function, was renamed RsmP for rod-shaped morphology protein.

RsmP Forms Filamentous Structures in Vivo—RsmP is a protein predicted to form coiled coils (44), and coiled-coil proteins are the main structural elements of many fibrous proteins in eukaryotes (41, 45) and prokaryotes (27). Therefore, based on

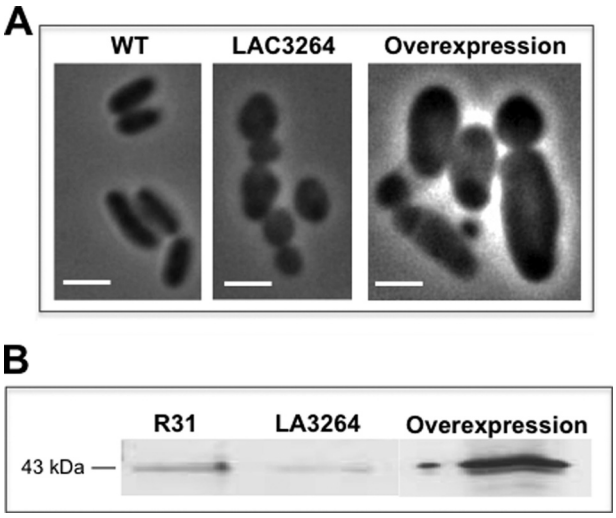

FIGURE 4. *A,* effect of RsmP depletion on cell shape and polar growth of *C. glutamicum*. Phase-contrast microscopy of exponentially growing *C. glutamicum* R31 (wild type), *C. glutamicum* LAC3264 cells, and *C. glutamicum* overexpressing RsmP are shown. Note that the typical morphology of *C. glutamicum* R31 was replaced by a coccoid morphology (no polar growth) in strain LAC3264 and replaced by club-shaped cells after overexpressing RsmP. *Bar* represents 1 μm. *B,* RsmP expression levels. The various RsmP levels of the *C. glutamicum* R31 (wild type), *C. glutamicum* LAC3264 cells, and *C. glutamicum* overexpressing RsmP were analyzed by immunoblotting using rabbit anti-RsmP antibodies. Cells were grown, harvested, and disrupted. Equal amounts (1 μg) of crude lysates were then loaded onto an acrylamide gel, subjected to electrophoresis, and transferred onto PVDF membranes for immunoblot analysis.

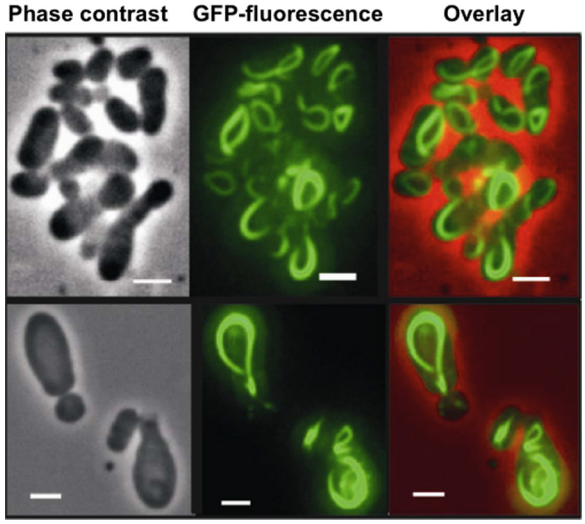

FIGURE 5. *Left panel,* phase contrast; *middle panel*, GFP fluorescence, and *right panel,* overlay of phase contrast and GFP fluorescence images of *C. glutamicum* carrying plasmid pE_RsmP-GFP. Note that the typical rod-shaped morphology of *C. glutamicum* was replaced by club-shaped cells, and RsmP-GFP is forming helical/filamentous structures along the cell.

the conditional mutant phenotype showing that RsmP (Cg3264) is involved in polar growth or rod-shaped determination, we hypothesized that RsmP could support cell shape by forming intracellular filaments or a bacterial cytoskeleton like CreS, FilP, or Ccrp (29, 30, 46). To test this hypothesis, we cloned *rsmP* into the *E. coli*/corynebacterial shuttle plasmid pEAG6 designed to easily clone and overexpress any promoterless gene under the control of the P*div* promoter and fused to *egfp2* (23), thus generating pE_RsmP-GFP. Then, when this plasmid was introduced into *C. glutamicum*, the cells showed a club-shaped morphology (Fig. 4*A*) similar to that in DivIVA-overexpressing strains (19, 23) and RsmP-GFP proteins localized as long filaments along the cell (Fig. 5). This suggested that RsmP formed helical/filamentous structures *in vivo* extending from one pole to the other at a cellular location consistent with a possible and essential cytoskeletal function, and therefore in accordance with our results showing that RsmP was indeed involved in determining rod-shaped morphology.

RsmP Forms Filamentous Structures in Vitro—RsmP was able to form filamentous structures *in vivo*, as observed with GFP localization. We therefore tried to confirm RsmP function as a cytoskeletal protein and whether RsmP could form filaments *in vitro*. First, we used a polyhistidine-tagged version of RsmP (His-RsmP) purified from *E. coli* by affinity chromatography, but most of the protein was present in inclusion bodies, a typical characteristic of IFs (45, 47). This was solved by overnight incubation of the overexpressing *E. coli* strain at 17 °C, and the soluble RsmP proteins were purified and used in an "*in vitro*" polymerization assay using the cross-linking agent DTBP widely used to study the polymerization of eukaryotic IF proteins (48–50). His-RsmP was incubated in the presence of DTBP, and the reaction was stopped after 15 or 30 min by adding 1 M Tris-HCl, pH 8. The reaction product was loaded onto an SDS-PAGE and detected by Western blot using commercial anti-histidine antibodies. As shown in Fig. 6*A*, His-RsmP formed dimers, trimers, and tetramers in the presence of DTBP. Furthermore, the polymerization reaction was reversed by adding 100–150 mM dithiothreitol (DTT) at 37 °C for 30 min, confirming the specificity of the polymerization reaction (Fig. 6*A*).

To further confirm the ability of RsmP to form filamentous structures *in vitro*, we used a complementary strategy. In fact, most IF proteins have the ability to form filaments *in vitro* without divalent cations, nucleotides, or other exogenous factors (47). Because of the high insolubility of IFs, *in vitro* assembly first requires the solubilization of the IF protein in a strong denaturating agent, and filaments are obtained after dialyzing the protein against physiological or low ionic strength buffers at a neutral pH (51). This was the method used to demonstrate that CreS from *C. crescentus* and FilP from *S. coelicolor* formed filamentous structures *in vitro* (28, 29). Therefore, we purified RsmP under strong denaturating conditions (8 M urea), and once purified, RsmP was spontaneously able to self-assemble into filaments after removal of urea by several dialysis steps. The filaments formed were confirmed by transmission electron microscopy (Fig. 7); they appeared similar to the filaments described for the Ccrp59 (coiled-coil rich proteins) from *H. pylori* (30). RsmP formed straight and branched filamentous structures measuring on average 2000 nm long and 50 nm diameter, thus definitively confirming its function as a cytoskeletal protein.

RsmP Is Preferentially Phosphorylated by the Ser/Thr Protein Kinase Cg_PknA—Eukaryotic IF proteins can occasionally be regulated post-translationally by glycosylation or phosphorylation (41, 52). Indeed, phosphorylation on their head and tail domains as well as the dynamics of their phosphorylation/dephosphorylation play a major role in regulating the assembly/

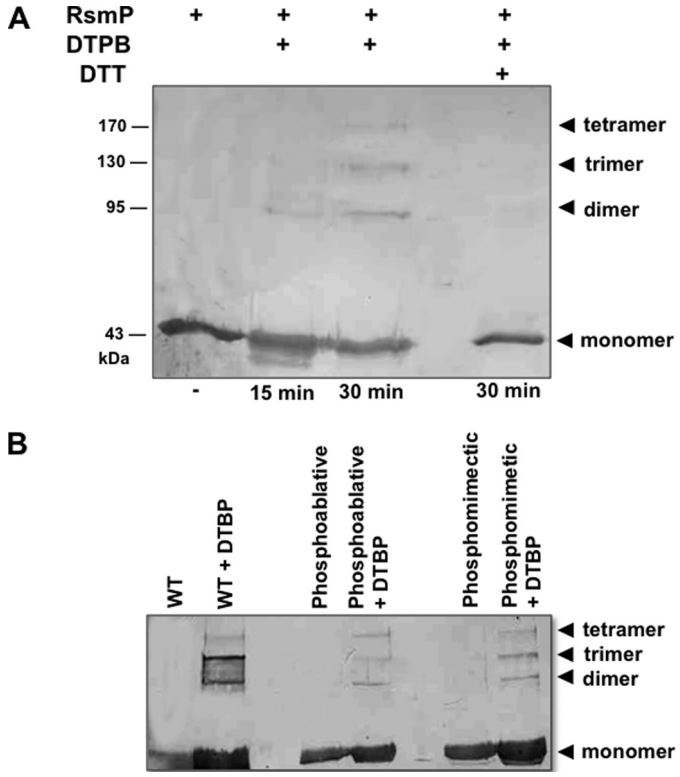

FIGURE 6. *A, in vitro* polymerization assay of the wild-type His-tagged RsmP from *C. glutamicum* using the cross-linking agent DTBP. Protein RsmP was incubated for 15 or 30 min in the presence of a 10 times molar excess of DTBP, and the reaction was stopped by adding 1 M Tris. Proteins were separated on a 12% SDS gel, electroblotted onto PVDF membranes (Millipore), and detected using anti-His antibodies. The specificity of the polymerization reaction was tested by treatment with 100 mM DTT. Note the formation of dimers, trimers, and tetramers in the presence of DTBP. *B, in vitro* polymerization assays of the His-tagged wild-type RsmP protein, the His-tagged RsmP protein S6A/T168A/T211A (phosphoablative protein), and the His-tagged RsmP protein S6D/T168D/T211D (phosphomimetic protein). Note that mutant proteins can also form dimers, trimers, and tetramers in the presence of DTBP.

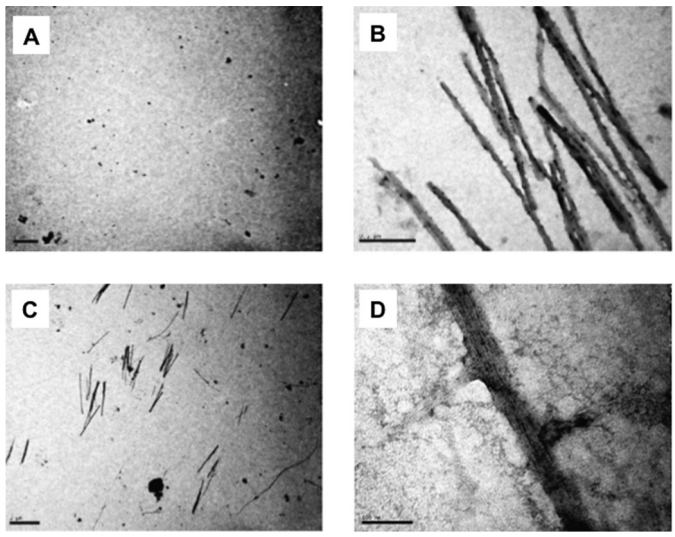

FIGURE 7. *A,* control with no protein. *B–D,* transmission electron micrographs of negatively stained filaments formed by purified RsmP. *Size bars* represent 2 μm for *A* and *C*, 0.5 μm for *B*, and 100 nm for *D*. The average size of the filaments is 2000 nm long and 50 nm diameter. Note the bundle of filaments in *D*.

disassembly of these filaments and consequently their functions (53). As RsmP shares characteristics of IF elements, we investigated whether RsmP could be post-translationally modified by phosphorylation via the STPKs present in the genome of *C. glutamicum* (33). The kinases Cg_PknA, Cg_PknB, and Cg_PknL from *C. glutamicum* were expressed as GST-tagged fusion proteins and purified from *E. coli* as reported earlier (33). Each of the purified kinases was incubated with RsmP and [γ-^{33}P]ATP and resolved by SDS-PAGE, and their phosphorylation profiles were analyzed by autoradiography. The presence of an intense radioactive signal indicated that RsmP was preferentially phosphorylated by Cg_PknA (Fig. 8*A*), and to a lesser extent by Cg_PknL, although no signal was observed in the presence of Cg_PknB. These results clearly indicated that RsmP is a specific substrate and interacts with *C. glutamicum* STPKs *in vitro*, suggesting that this key protein controlling rod-shaped morphology might be regulated in corynebacteria by multiple extracellular signals.

RsmP Is Phosphorylated on Ser and Thr Residues—To identify which residues of RsmP corresponded to the phosphorylated site(s), a mass spectrometric approach was used. RsmP was incubated with cold ATP in the presence of Cg_PknA (the most active kinase for RsmP, see Fig. 8*A*) and subjected to mass spectrometric analysis after tryptic and chymotryptic digestion, as described previously (34). Analysis of tryptic and chymotryptic digests allowed the characterization of three phosphorylation sites in RsmP corresponding to Ser-6, Thr-168, and Thr-211, which lie outside the coiled-coil regions (Table 3 and Fig. 3). Definitive identification of the Ser-6, Thr-168, and Thr-211 residues identified by mass spectrometry was achieved by successive site-directed mutagenesis by introducing mutations that prevented their specific phosphorylation. Thus, all three residues were replaced by alanine, yielding the mutant RsmP_S6A/T168/T211A. This mutant protein (phosphoablative mutant) was expressed in *E. coli*, purified, and incubated with [γ-^{33}P]ATP and Cg_PknA (Fig. 8*B*). Phosphorylation of the phosphoablative mutant protein appeared to be completely abolished, confirming the identification of all three sites of phosphorylation. An additional round of mass spectrometric analysis was also performed directly on the Ala mutant protein, which failed to identify any additional phosphate group.

RsmP Phosphorylation Negatively Regulates Filament Formation—Phosphorylation of IFs have been reported to block vimentin and desmin polymerization *in vitro* as well as to disassemble pre-existing filaments (54, 55). To investigate the consequences of phosphorylation on RsmP filament forming activity, Ser-6, Thr-168, and Thr-211 residues were replaced by aspartic acid residues able to mimic the phosphorylated isoform of the protein, generating the RsmP_S6D/T168D/211D protein (phosphomimetic mutant) (56–59). The three RsmP alleles (wild-type, phosphoablative, or phosphomimetic) were expressed in *E. coli*, purified, and used in *in vitro* cross-linking experiments. As showed in Fig. 6*B*, the three proteins could form dimers, trimers, and tetramers in the presence of DTBP, suggesting that phosphorylation did not affect the polymerization activity of RsmP. However, for the eukaryotic IF vimentin, phosphorylation in the N-terminal head domain induced disassembly of vimentin filaments and resulted in the release of tetrameric subunits (60), thus leading to the conclusion that

phosphorylation does not affect the formation of tetramers but the assembly of tetramers into filaments. Therefore, we hypothesized that RsmP might behave in a similar way. The three alleles were cloned into pEAG6 to obtain the three enhanced GFP fusions, which were introduced into *C. glutamicum* and observed by fluorescence microscopy. As shown in Fig. 9, RsmP, corresponding to the phosphomimetic protein, leads to the loss of protein localization along the cell and concentration at the poles, although the phosphoablative mutant showed a similar localization as in the wild type. These results demonstrate that modification of the phosphorylation sites by phosphomimetic residues affects localization in a manner consistent with control of localization by phosphorylation and thus confirmed the critical role of phosphorylation in regulating RsmP localization within the cell.

DISCUSSION

In all actinobacteria studied, cell elongation occurs at the cell poles and is supported by the coiled coil-rich protein DivIVA (19–23). This protein is regulated by phosphorylation in *M. tuberculosis* (22, 57, 61) but not in *C. glutamicum*, suggesting that corynebacteria uses different cell elongation control mechanisms. In this study, we characterized a novel protein, conserved almost exclusively in all *Corynebacterium* species and overexpressed in the DivIVA-depleted strain. This protein, renamed here RsmP, corresponds to a coiled coil-rich protein with a central rod domain of two coiled-coil segments flanked by head and tail domains. This structural organization is similar to those of DivIVA, CreS, and FilP proteins and resembles the organization of eukaryotic intermediate filaments. In addition, RsmP can polymerize to form filamentous structures *in vitro* without the addition of any external cofactors, and *in vivo* the RsmP structures extend from one cell pole to the other like CreS. The structural organization of RsmP, the partial insolubility of this protein, and its ability to polymerize spontaneously are archetypical characteristics of eukaryotic intermediate filaments. Moreover, this protein is essential to *C. glutamicum* cell viability, with overexpression and partial depletion of the gene producing severe morphological alterations, thus indicating that RsmP is involved in maintenance of corynebacterial cell shape.

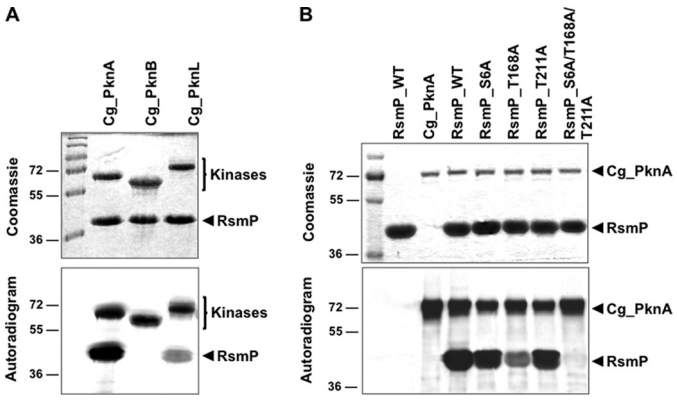

FIGURE 8. *A, in vitro* phosphorylation of *C. glutamicum* RsmP by corynebacterial STPKs. The recombinant STPKs (Cg_PknA, Cg_PknB, and Cg_PknL) were expressed and purified as described previously (33). Recombinant RsmP was treated with the tobacco etch virus protease to remove the N-terminal His tag and then incubated with [γ-^{33}P]ATP and the different kinases. Samples were separated by SDS-PAGE, Coomassie-stained (*upper panel*), and visualized by autoradiography (*lower panel*). *Upper bands* illustrate the autokinase activity of each STPK, and *lower bands* reflect RsmP phosphorylation. *B, in vitro* phosphorylation of RsmP mutants by Cg_PknA. The different RsmP mutants were treated with the tobacco etch virus protease to remove the N-terminal His tag and then used in phosphorylation assays in equal amounts in the presence of [γ-^{33}P]ATP and Cg_PknA. The RsmP_WT, RsmP_S6A, RsmP_T168A, RsmP_T211A, and RsmP_S6A/T168A/T211A mutant proteins were separated by SDS-PAGE and stained with Coomassie Blue (*upper panel*), and the radioactive bands were revealed by autoradiography (*lower panel*).

In eukaryotic cells, intermediate filaments are usually controlled by different post-translational modifications such as phosphorylation. In previous reports, we have characterized the four STPKs of *C. glutamicum* and demonstrated that the PknA and PknB kinases were involved in cell division and PG biosynthesis. Moreover, different STPK substrates have been identified and characterized as proteins involved in cell division and PG biosynthesis thus confirming the critical role of Ser/Thr phosphorylation in regulating such pathways (34, 62). Therefore, we hypothesized that RsmP could be regulated via STPK phosphorylation, and this was confirmed in this study by evidence that corynebacterial PknA and PknL can phosphorylate RsmP. To our knowledge, this is the first study of a bacterial IF-like protein being regulated by phosphorylation as none of the prokaryotic intermediate filament-like proteins (FilP, CreS, and Ccrp) were described previously as being phosphorylated.

In vitro phosphorylation assays coupled with mass spectrometric analysis led us to identify three phosphorylated residues in RsmP. None of them is located in the coiled-coil regions, and apparently their replacement does not affect the RsmP polymerization ability, as it is the case with eukaryotic intermediate filaments, where phosphorylation prevents polymerization of tetramers into filaments (31). Determination of the phosphorylation sites provided the essential groundwork for mechanistic/functional studies on RsmP and demonstrated the efficacy of combining genetics and mass spectrometric analyses with precise identification of phosphoacceptors, a prerequisite for a further understanding of the RsmP regulation. Moreover, strains with defined mutations within the phosphorylation sites will be extremely helpful in establishing the role of RsmP phosphorylation-dependent regulation in corynebacterial growth and cell division, and in fact, the localization pattern of RsmP is affected by phosphorylation. Whereas overexpression

TABLE 3

Sequence of the phosphorylated peptides identified in Cg3264 as determined by mass spectrometry

Phosphorylated residues are indicated with a p. M_{ox} corresponds to an oxidized methionine. Residues originating from the His tag are underlined.

Phosphorylated tryptic and chymotryptic peptide sequence	No. of detected phosphate groups, LC-ESI/MS/MS	Phosphorylated residue(s)
((−3)−7) GSHMANPL**pS**K	1	Ser-6
((−3)−7) GSHMANPL**pS**KGWK	1	Ser-6
(163–191) MQESV**pT**KSM$_{ox}$DSLNQFGTQDSSVPTLDAVR	1	Thr-168
(163–191) MQESV**pT**KSMDSLNQFGTQDSSVPTLDAVR	1	Thr-168
(198–215) YADALGAQELTQN**pT**VSDR	1	Thr-211

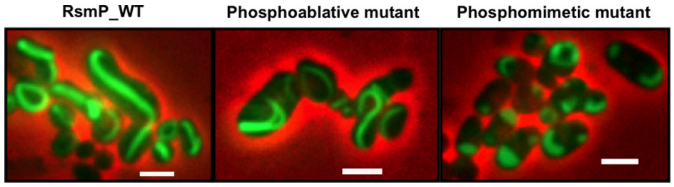

FIGURE 9. **GFP fluorescence *C. glutamicum* cells carrying plasmid pE_RsmP-GFP (wild-type), pE_RsmPA-GFP (phosphoablative), and pE_RsmPD-GFP (phosphomimetic).** Note that the club-shaped cells of *C. glutamicum* carrying either the plasmid containing the wild-type or phosphoablative RsmP-GFP were replaced by rod-shaped cells in *C. glutamicum* carrying the phosphomimetic RsmP-GFP.

of the wild-type protein and the phosphoablative mutant exhibit a similar localization forming an internal cytoskeleton extending from one cell pole to the other, overexpression of the phosphomimetic mutant results in localization predominantly at the cells poles.

In light of these data, we propose two possible roles for RsmP in *C. glutamicum* cell elongation. First, RsmP may be able to polymerize from pole to pole to generate an internal scaffold that supports the lateral wall during cell elongation, as it is the case in *B. subtilis* or *E. coli* where this function is carried out by actin-like MreB homologues (6, 63). Therefore, MreB function may be provided by IF-like proteins in corynebacteria whose genomes lack actin-like homologues (15–18). Phosphorylation may then be involved in the control of RsmP polymerization and subsequently in cell elongation. *C. glutamicum* has a well known bacillary-to-coccoid pleomorphism during late exponential growth phase (64). This pleomorphism has been linked to *bolA* in *E. coli* (65, 66), but to date no other molecular factors have been identified that would explain the cell-shape shift in response to nutrient deprivation in other bacteria. Therefore, PknA and PknL may phosphorylate RsmP under these conditions to produce a larger number of smaller cells to distribute the stress caused by starvation over a larger number of individuals, increasing the probability of survival of at least a few of them (67).

Alternatively, RsmP may be another element of the cytoskeletal structure recruited at the cell poles by DivIVA, which directs PG synthesis for cell elongation in actinobacteria. In this hypothesis, RsmP would provide an additional internal support, probably by protein-protein interaction through its coiled-coil domains. Therefore, RsmP may be the substrate of the Pkn-mediated signal transduction of the polar PG synthesis complex in *C. glutamicum*. Indeed, the corynebacteria-specific conservation of RsmP, the striking lack of DivIVA phosphorylation in corynebacteria (well documented in other actinomycetes), the up-regulation of RsmP in DivIVA-depleted strains, and the polar localization of the phosphomimetic mutant support a corynebacteria-specific role of RsmP in polar PG synthesis.

In conclusion, the data reported here provide significant and novel insight into the bacterial cytoskeleton and function of cytoskeletal elements. Especially significant, this study confirmed that *C. glutamicum* possesses an original and specific system for establishing and maintaining rod-shaped morphology. Furthermore, these findings could be useful to extend our present understanding in cell division and cell-shape determination in Gram-positive bacteria toward the design of new antimicrobial drugs. The essential protein RsmP could be a useful target to combat emergent corynebacterial pathogens, especially the human pathogen *C. diphtheriae*.

Acknowledgments—We thank M. Becchi and A. Cornut (Institut de Biologie et Chimie des Protéines, Lyon, France) for excellent technical assistance in mass spectrometric analysis and David Hopwood for critical reading of the manuscript.

REFERENCES

1. Cabeen, M. T., and Jacobs-Wagner, C. (2005) *Nat. Rev. Microbiol.* **3,** 601–610
2. Scheffers, D. J., and Pinho, M. G. (2005) *Microbiol. Mol. Biol. Rev.* **69,** 585–607
3. Shih, Y. L., and Rothfield, L. (2006) *Microbiol. Mol. Biol. Rev.* **70,** 729–754
4. Margolin, W. (2005) *Nat. Rev. Mol. Cell Biol.* **6,** 862–871
5. Doi, M., Wachi, M., Ishino, F., Tomioka, S., Ito, M., Sakagami, Y., Suzuki, A., and Matsuhashi, M. (1988) *J. Bacteriol.* **170,** 4619–4624
6. Defeu Soufo, H. J., and Graumann, P. L. (2004) *EMBO Rep.* **5,** 789–794
7. Figge, R. M., Divakaruni, A. V., and Gober, J. W. (2004) *Mol. Microbiol.* **51,** 1321–1332
8. de Pedro, M. A., Quintela, J. C., Höltje, J. V., and Schwarz, H. (1997) *J. Bacteriol.* **179,** 2823–2834
9. Carballido-López, R. (2006) *Microbiol. Mol. Biol. Rev.* **70,** 888–909
10. Daniel, R. A., and Errington, J. (2003) *Cell* **113,** 767–776
11. Leaver, M., and Errington, J. (2005) *Mol. Microbiol.* **57,** 1196–1209
12. Kruse, T., Bork-Jensen, J., and Gerdes, K. (2005) *Mol. Microbiol.* **55,** 78–89
13. Divakaruni, A. V., Baida, C., White, C. L., and Gober, J. W. (2007) *Mol. Microbiol.* **66,** 174–188
14. Umeda, A., and Amako, K. (1983) *Microbiol. Immunol.* **27,** 663–671
15. Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B. J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Krämer, R., Linke, B., McHardy, A. C., Meyer, F., Möckel, B., Pfefferle, W., Pühler, A., Rey, D. A., Rückert, C., Rupp, O., Sahm, H., Wendisch, V. F., Wiegräbe, I., and Tauch, A. (2003) *J. Biotechnol.* **104,** 5–25
16. Nishio, Y., Nakamura, Y., Kawarabayasi, Y., Usuda, Y., Kimura, E., Sugimoto, S., Matsui, K., Yamagishi, A., Kikuchi, H., Ikeo, K., and Gojobori, T. (2003) *Genome Res.* **13,** 1572–1579
17. Cerdeño-Tárraga, A. M., Efstratiou, A., Dover, L. G., Holden, M. T., Pallen, M., Bentley, S. D., Besra, G. S., Churcher, C., James, K. D., De Zoysa, A., Chillingworth, T., Cronin, A., Dowd, L., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Moule, S., Quail, M. A., Rabbinowitsch, E., Rutherford, K. M., Thomson, N. R., Unwin, L., Whitehead, S., Barrell, B. G., and Parkhill, J. (2003) *Nucleic Acids Res.* **31,** 6516–6523
18. Tauch, A., Kaiser, O., Hain, T., Goesmann, A., Weisshaar, B., Albersmeier, A., Bekel, T., Bischoff, N., Brune, I., Chakraborty, T., Kalinowski, J., Meyer, F., Rupp, O., Schneiker, S., Viehoever, P., and Pühler, A. (2005) *J. Bacteriol.* **187,** 4671–4682
19. Ramos, A., Honrubia, M. P., Valbuena, N., Vaquera, J., Mateos, L. M., and Gil, J. A. (2003) *Microbiology* **149,** 3531–3542
20. Flärdh, K. (2003) *Mol. Microbiol.* **49,** 1523–1536
21. Nguyen, L., Scherr, N., Gatfield, J., Walburger, A., Pieters, J., and Thompson, C. J. (2007) *J. Bacteriol.* **189,** 7896–7910
22. Kang, C. M., Nyayapathy, S., Lee, J. Y., Suh, J. W., and Husson, R. N. (2008) *Microbiology* **154,** 725–735
23. Letek, M., Ordóñez, E., Vaquera, J., Margolin, W., Flärdh, K., Mateos, L. M., and Gil, J. A. (2008) *J. Bacteriol.* **190,** 3283–3292
24. Muchová, K., Kutejová, E., Scott, D. J., Brannigan, J. A., Lewis, R. J., Wilkinson, A. J., and Barák, I. (2002) *Microbiology* **148,** 807–813
25. Stahlberg, H., Kutejová, E., Muchová, K., Gregorini, M., Lustig, A., Müller, S. A., Olivieri, V., Engel, A., Wilkinson, A. J., and Barák, I. (2004) *Mol. Microbiol.* **52,** 1281–1290
26. Wang, S. B., Cantlay, S., Nordberg, N., Letek, M., Gil, J. A., and Flärdh, K. (2009) *FEMS Microbiol. Lett.* **297,** 101–109

27. Graumann, P. L. (2009) *Cell Motil. Cytoskeleton* **66,** 909–914
28. Ausmees, N., Kuhn, J. R., and Jacobs-Wagner, C. (2003) *Cell* **115,** 705–713
29. Bagchi, S., Tomenius, H., Belova, L. M., and Ausmees, N. (2008) *Mol. Microbiol.* **70,** 1037–1050
30. Waidner, B., Specht, M., Dempwolff, F., Haeberer, K., Schaetzle, S., Speth, V., Kist, M., and Graumann, P., L. (2009) **5,** e1000669
31. Parry, D. A., Strelkov, S. V., Burkhard, P., Aebi, U., and Herrmann, H. (2007) *Exp. Cell Res.* **313,** 2204–2216
32. Mateos, L. M., Schäfer, A., Kalinowski, J., Martin, J. F., and Pühler, A. (1996) *J. Bacteriol.* **178,** 5768–5775
33. Fiuza, M., Canova, M. J., Zanella-Cléon, I., Becchi, M., Cozzone, A. J., Mateos, L. M., Kremer, L., Gil, J. A., and Molle, V. (2008) *J. Biol. Chem.* **283,** 18099–18112
34. Fiuza, M., Canova, M. J., Patin, D., Letek, M., Zanella-Cléon, I., Becchi, M., Mateos, L. M., Mengin-Lecreulx, D., Molle, V., and Gil, J. A. (2008) *J. Biol. Chem.* **283,** 36553–36563
35. Daniel, R. A., Harry, E. J., and Errington, J. (2000) *Mol. Microbiol.* **35,** 299–311
36. Mengin-Lecreulx, D., Ayala, J., Bouhss, A., van Heijenoort, J., Parquet, C., and Hara, H. (1998) *J. Bacteriol.* **180,** 4406–4412
37. Elderkin, S., Jones, S., Schumacher, J., Studholme, D., and Buck, M. (2002) *J. Mol. Biol.* **320,** 23–37
38. Darwin, A. J. (2005) *Mol. Microbiol.* **57,** 621–628
39. Jones, D. T. (1999) *J. Mol. Biol.* **292,** 195–202
40. Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M., and Kim, P. S. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 8259–8263
41. Herrmann, H., and Aebi, U. (2004) *Annu. Rev. Biochem.* **73,** 749–789
42. Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N., and Schoner, B. E. (1992) *Gene* **116,** 43–49
43. Letek, M., Valbuena, N., Ramos, A., Ordóñez, E., Gil, J. A., and Mateos, L. M. (2006) *J. Bacteriol.* **188,** 409–423
44. Lupas, A., Van Dyke, M., and Stock, J. (1991) *Science* **252,** 1162–1164
45. Herrmann, H., Bär, H., Kreplak, L., Strelkov, S. V., and Aebi, U. (2007) *Nat. Rev. Mol. Cell Biol.* **8,** 562–573
46. Ausmees, N. (2006) *J. Mol. Microbiol. Biotechnol.* **11,** 152–158
47. Domingo, A., Sarria, A. J., Evans, R. M., and Klymkowsky, M. W. (1992) *The Cytoskeleton: A Practical Approach* (Carraway, K. L., and Carraway, C. A. C., eds) pp. 223–255, IRL Press at Oxford University Press, Oxford, United Kingdom
48. Downing, D. T. (1996) *Proteins* **25,** 215–224
49. Steinert, P. M., Marekov, L. N., Fraser, R. D., and Parry, D. A. (1993) *J. Mol. Biol.* **230,** 436–452
50. Charulatha, V., and Rajaram, A. (2001) *J. Biomed. Mater. Res.* **54,** 122–128
51. Steinert, P. M., Idler, W. W., and Zimmerman, S. B. (1976) *J. Mol. Biol.* **108,** 547–567
52. Chou, S. M., and Wang, H. S. (1997) *J. Neurol. Sci.* **152,** 198–209
53. Sihag, R. K., Inagaki, M., Yamaguchi, T., Shea, T. B., and Pant, H. C. (2007) *Exp. Cell Res.* **313,** 2098–2109
54. Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M., and Sato, C. (1987) *Nature* **328,** 649–652
55. Geisler, N., and Weber, K. (1988) *EMBO J.* **7,** 15–20
56. Cohen-Gonsaud, M., Barthe, P., Canova, M. J., Stagier-Simon, C., Kremer, L., Roumestand, C., and Molle, V. (2009) *J. Biol. Chem.* **284,** 19290–19300
57. Kang, C. M., Abbott, D. W., Park, S. T., Dascher, C. C., Cantley, L. C., and Husson, R. N. (2005) *Genes Dev.* **19,** 1692–1704
58. Veyron-Churlet, R., Molle, V., Taylor, R. C., Brown, A. K., Besra, G. S., Zanella-Cléon, I., Fütterer, K., and Kremer, L. (2009) *J. Biol. Chem.* **284,** 6414–6424
59. Veyron-Churlet, R., Zanella-Cléon, I., Cohen-Gonsaud, M., Molle, V., and Kremer, L. (2010) *J. Biol. Chem.* **285,** 12714–12725
60. Eriksson, J. E., He, T., Trejo-Skalli, A. V., Härmälä-Braskén, A. S., Hellman, J., Chou, Y. H., and Goldman, R. D. (2004) *J. Cell Sci.* **117,** 919–932
61. Hamasha, K., Sahana, M. B., Jani, C., Nyayapathy, S., Kang, C. M., and Rehse, S. J. (2010) *Biochem. Biophys. Res. Commun.* **391,** 664–668
62. Schultz, C., Niebisch, A., Schwaiger, A., Viets, U., Metzger, S., Bramkamp, M., and Bott, M. (2009) *Mol. Microbiol.* **74,** 724–741
63. Vats, P., Shih, Y. L., and Rothfield, L. (2009) *Mol. Microbiol.* **72,** 170–182
64. Fuhrmann, C., Soedarmanto, I., and Lämmler, C. (1997) *Zentralbl. Veterinarmed. B.* **44,** 287–294
65. Santos, J. M., Freire, P., Vicente, M., and Arraiano, C. M. (1999) *Mol. Microbiol.* **32,** 789–798
66. Freire, P., Moreira, R. N., and Arraiano, C. M. (2009) *J. Mol. Biol.* **385,** 1345–1351
67. James, G. A., Korber, D. R., Caldwell, D. E., and Costerton, J. W. (1995) *J. Bacteriol.* **177,** 907–915
68. Saitou, N., and Nei, M. (1987) *Mol. Biol. Evol.* **4,** 406–425
69. Zuckerkandl, E., and Pauling, L. (1965) *Evolving Genes and Proteins,* pp. 97–166, Academic Press, New York
70. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) *Mol. Biol. Evol.* **24,** 1596–1599
71. Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A. H., and Pühler, A. (1990) *J. Bacteriol.* **172,** 1663–1666
72. Santamaria, R. I., Gil, J. A., and Martin, J. F. (1985) *J. Bacteriol.* **162,** 463–467
73. Canova, M. J., Kremer, L., and Molle, V. (2008) *Plasmid* **60,** 149–153